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Chlorophyll Breakdown in Senescent Chloroplasts[1]

Cleavage of Pheophorbide *a* in Two Enzymic Steps

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The cleavage of pheophorbide (Pheide) *a* into primary fluorescent chlorophyll (Chl) catabolites (pFCCs) in senescent chloroplasts was investigated. Chloroplast preparations isolated from senescent canola (*Brassica napus*) cotyledons exhibited light-dependent production of pFCC when assay mixtures were supplemented with ferredoxin (Fd). pFCC production in detergent-solubilized membranes was dependent on the presence of an Fd-reducing system. Pheide *a* cleavage required the action of two proteins, Pheide *a* oxygenase and a stroma protein. In the absence of stroma protein, Pheide *a* oxygenase converted Pheide *a* into a red Chl catabolite (RCC), the presumptive intermediary product of Pheide *a* cleavage. Incubation of the stroma protein (RCC reductase) together with chemically synthesized RCC resulted in the production of three different FCCs. Two of these catabolites were identical to the pFCCs from canola or barley (*Hordeum vulgare*) (pFCC-1) and sweet pepper (*Capsicum annuum*) (pFCC-2), respectively. Thus, the conversion of Pheide *a* to pFCC could be demonstrated to proceed in two consecutive steps, and both reactions depended on reduced Fd as the source of electrons. The function of Fd in Chl breakdown in vivo is corroborated by the marked retention of this protein until the late stages of senescence, as demonstrated by immunoblotting.

The metabolism of Chl has so far been investigated primarily with regard to biosynthesis, whereas breakdown has been largely neglected. Despite some recent progress in the identification of breakdown products and enzymes engaged in the degreening of senescent leaves (for a recent review, see Matile et al., 1996), important details of the catabolic pathway are still missing. In particular, an oxygenolytic cleavage of the porphyrin moiety was indicated by the first structure of a colorless Chl catabolite (Kräutler et al., 1991), but the enzymic reaction responsible for it has so far only been partially characterized. This key reaction of the breakdown pathway was initially thought to take place in isolated intact gerontoplasts (senescent chloroplasts; Sitte et al., 1980). It has since been shown to depend on dioxygen and to yield blue FCCs (Schellenberg et al., 1990; Matile et al., 1992; Ginsburg et al., 1994). Subsequent work with gerontoplast membranes has led to the identification of an Fe-containing oxygenase that specifically recognizes Pheide *a* as a substrate and produces an FCC; the reaction in vitro required dioxygen and depended on reduced Fd as well as the presence of stroma protein (Ginsburg et al., 1994; Hörtensteiner et al., 1995). The oxygenase has been localized in the envelope of gerontoplasts (Matile and Schellenberg, 1996; Matile et al., 1997).

In senescent leaves FCCs are further metabolized into NCCs. Whereas the structure of the NCCs so far determined in senescent primary leaves of barley (*Hordeum vulgare*) and canola (*Brassica napus*) cotyledons exhibits species-specific differences (Matile et al., 1996), the first products of Pheide *a* cleavage in vitro, *Hv*-FCC-4 (barley) and *Bn*-FCC-2 (canola), appear to be identical, as judged by their retention times on reverse-phase HPLC. Recently, the structure of this apparently primary catabolite of Pheide *a* has been determined (Fig. 1; Mühlecker et al., 1997). The conversion of Pheide *a* into *Bn*-FCC-2 formally involves the introduction of two atoms of oxygen (opening of the macrocycle in the C4/C5 mesoposition) as well as of four atoms of hydrogen (reduction of double bonds of the β- and δ-methine bridges). Therefore, there is little doubt that the reaction must proceed in more than a single step. This is also suggested by the finding that the reaction takes place only in the presence of protein from both gerontoplast membranes and the stroma. However, to our knowledge, it has thus far not been possible to establish the function of the stromal factor or to detect an intermediary catabolite produced by the membrane-associated oxygenase in the absence of stroma protein.

Knowledge of the constitution of the fluorescent catabolite *Bn*-FCC-2 provided a solid structural basis for a convincing working model involving a linear red tetrapyrrole as a possible, or even likely, intermediary catabolite of the PaO system, as depicted in Figure 1 (Mühlecker et al., 1997). Structurally related compounds have been identified as an isolation form of the final product of Chl breakdown

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Abbreviations: Chl, chlorophyll; FCC, fluorescent Chl catabolite; FNR, Fd-NADPH oxidoreductase; FU, fluorescence units; Glc6P, Glc-6 phosphate; Glc6P-DH, Glc-6-phosphate dehydrogenase; NCC, nonfluorescent Chl catabolite; PaO, pheophorbide *a* oxygenase; Pheide, pheophorbide; RCC, red Chl catabolite.

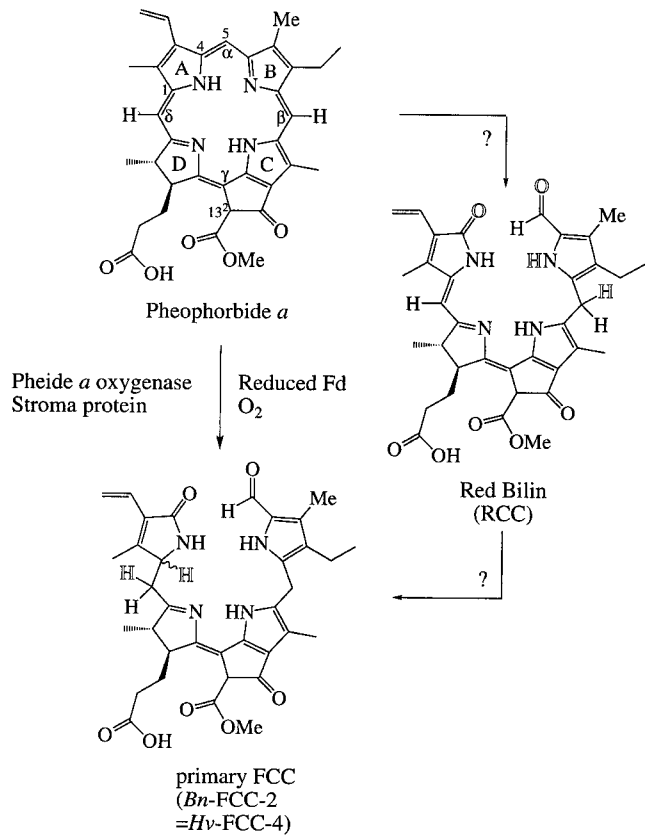

Figure 1. Structures of Pheide *a* and its product, a primary FCC, after cleavage by PaO and stroma protein. A possible intermediate of this reaction, RCC, is also depicted. The changes in the chemical structures are outlined. Note the numbering of carbon atoms, methine bridges (α to δ), and pyrrols (A–D), as mentioned in the text.

in *Chlorella protothecoides* (Engel et al., 1996). Unlike higher plants, which accumulate the final products of Chl breakdown in the vacuoles of senescent mesophyll cells (Hinder et al., 1996), this alga releases red bilins into the culture medium when bleaching of the cells is induced under appropriate conditions.

Studies on Chl breakdown in *C. protothecoides* employing ^{18}O-dioxygen have shown that the red bilin derived from Chl *a* is the product of monooxygenase-catalyzed oxygenation suggested to involve the C4/C5 double bond and to be followed by hydrolysis of the intermediary epoxide and subsequent ring opening through intramolecular rearrangement (Gossauer, 1994; Curty et al., 1995). Such a mechanism of ring opening of the Chl macrocycle would account for the introduction of two atoms of oxygen and two atoms of hydrogen into the Pheide *a* molecule. Hence, a second step appeared likely to occur in senescent chloroplasts of plants, which would result in the reduction of an additional double bond at the unsubstituted δ-mesoposition (Fig. 1; Mühlecker et al., 1997). We hypothesize that this second step is catalyzed by the stroma protein required for in vitro conversion of Pheide *a* into the primary FCC. To test this hypothesis, a red bilin, RCC (for a nomenclature of Chl catabolites, see Ginsburg and Matile, 1993), was synthesized from Pheide *a* methyl ester (Kräutler et al., 1997) and employed as a substrate of the stroma protein. Moreover, we have attempted to identify RCC as an intermediate in the conversion of Pheide *a* into FCC in vitro.

MATERIALS AND METHODS

Cultivation of Plants and Senescence Induction

Barley (*Hordeum vulgare* L. cv Express) and canola (*Brassica napus* L. cv Arabella) (VOLG Agricultural Cooperative, Winterthur, Switzerland) seeds were germinated and grown in a growth chamber at 16°C during the dark period (12 h) and at 21°C during the light period (12 h; 120 W m^{-2}), with a RH of 70%. After 10 d senescence was induced by incubating excised shoots of canola and barley placed in flasks filled with tap water in permanent darkness for 4 and 6 d, respectively.

Preparation of Gerontoplast Membrane Proteins

Gerontoplast membranes were isolated and solubilized from senescent canola cotyledons as described previously (Hörtensteiner et al., 1995). PaO was partially purified from solubilized membranes of canola. Solubilized gerontoplast membranes from 170 g of senescent canola cotyledons were applied to an EAH-activated Sepharose column (6-mL bed volume; Pharmacia) equilibrated with 25 mM Tris-Mes, pH 8.0, 0.1% Triton X-100. After loading the column was washed with the same buffer containing 100 mM potassium chloride (10 bed volumes). PaO was subsequently eluted with 200 mM potassium chloride dissolved in the equilibration buffer. Fractions containing activity were pooled and desalted on Sephadex G25 fine (Pharmacia).

Preparation of Soluble Proteins

Senescent barley primary leaves were blended in a mixer (Omni, Sorvall) twice for 5 s in a medium (3 mL g^{-1} fresh weight) containing 25 mM Tricine-Tris, pH 8.0, 10 mM DTT, and 5 mM EDTA. After filtration through two layers of 25-μm nylon gauze, particulate material was removed by centrifugation (27,500*g*, 15 min). The fraction of the supernatant precipitating between 50 and 80% saturation with ammonium sulfate was employed for the preparation of soluble proteins containing the required stroma protein. The precipitate was dissolved in 10 mM potassium phosphate buffer, pH 7.0, 10 mM DTT, and desalted on Sephadex G50 fine. Protein extracts from the exocarp of *Capsicum annuum* were prepared as described by Moser and Matile (1997).

Assay for Production of FCCs from Pheide *a*

The assay mixtures contained the equivalent of 0.5 g fresh weight of gerontoplast membrane protein from senescent canola cotyledons and soluble proteins. The latter represented either the first supernatant (S1) obtained upon washing gerontoplast membranes from canola cotyledons (Hörtensteiner et al., 1995) or the 50 to 80% ammonium

sulfate fraction from barley primary leaves. For incubations in the light, nonsolubilized gerontoplast membranes were also employed. The complete assay mixture contained 0.5 mM Pheide *a* as a substrate and 1.5 mM NADPH, 2.5 mM Glc6P, 10 milliunits of Glc6P-DH, and 10 μg of Fd as cofactors. The total volume was 40 μL. After incubation for 30 min at room temperature in the dark or in the light, the reaction was terminated by the addition of 90 μL of methanol.

Assay for the Formation of FCCs from RCC

The complete assay mixture (50 μL) contained the 50 to 80% ammonium sulfate protein fraction from barley, equivalent to 0.4 g fresh weight, 10 μg of RCC (Kräutler et al., 1997), 0.6 mM NADPH, 2 mM Glc6P, 10 milliunits of Glc6P-DH, and 12 μg of Fd. Before incubation at room temperature in the dark, the sealed glass tube was flooded through syringes inserted into the septum with nitrogen for 5 min to remove the air. After incubation for 1 h, the reaction was stopped by the addition of 40 μL of methanol.

Assay for the Formation of RCC from Pheide *a*

Incubations were for 1 h in a volume of 125 μL containing the partially purified PaO of canola equivalent to 2 g of fresh weight, 0.3 mM Pheide *a*, 1.5 mM NADPH, 2.5 mM Glc6P, 10 mU of Glc6P-DH, 10 μg of Fd, and 20 milliunits of FNR. After incubation in the dark at room temperature, 170 μL of methanol was added to terminate the reaction. For incubations under anaerobic conditions, the reaction mixture was placed in a sealed glass tube and flooded with nitrogen for 5 min prior to incubation.

HPLC and Identification of Reaction Products

Methanolic extracts of assay mixtures were chromatographed in an HPLC system as described previously (Ginsburg et al., 1994). The reverse-phase column (ODS, Hypersil, Holliston, MA) was eluted isocratically (1 mL min^{-1}) with 67.5% (v/v) methanol and 32.5% (v/v) 0.1 M potassium buffer, pH 7.0, as a solvent. The consecutive monitoring of A_{320} (for FCCs and RCC) and fluorescence (at 320/450 for FCCs) allowed the identification of reaction products by their relative retention times. Standard preparations of the respective catabolites were used as references. Identifications were also confirmed by online monitoring of absorption spectra and comparison with the spectra of the respective standards.

The products of the stroma protein reaction (RCC to FCCs) were identified as *Bn*-FCC-2 (*B. napus*), which eluted after 5.9 min, and *Ca*-FCC-2 (*C. annuum*), which eluted after 7.1 min, both of which have absorption maxima at 320 and 360 nm (Moser and Matile, 1997; Mühlecker et al., 1997). A third fluorescent compound with a retention time of 5.4 min had the conspicuous spectrum of an FCC. Amounts of FCCs are given as integrated FUs.

The product of the PaO reaction (Pheide *a* to RCC) was identified as RCC by virtue of its relative retention time of 15.8 min (identical to standard RCC) and its typical absorption spectrum with maxima at 320 and 485 nm. Amounts of RCC are expressed as relative absorption peak heights. With the solvent used, Pheide *a* was not eluted from the column.

Electrophoresis and Immunoblotting

Leaf tissue was homogenized at 4°C with sand and 50 mM sodium-phosphate buffer, pH 7.2, 120 mM 2-mercaptoethanol, 1 mM PMSF, and 5% (w/v) glycerol (9 mL g^{-1} fresh weight). SDS was added to a final concentration of 2%. An aliquot was removed for determination of Chl (Lichtenthaler, 1987) and protein (Bradford, 1976), and the remaining homogenate, after boiling for 5 min and subsequent centrifugation, was directly employed for SDS-PAGE (Laemmli, 1970). After transfer of proteins to a nylon membrane (Towbin et al., 1979), a sheep antiserum to Fd from *Festuca pratensis* (Mort et al., 1992) was employed for immuno-identification of Fd.

Preparation of Fd, Pheide *a*, and RCC

Fd was isolated from spinach leaves and Pheide *a* was prepared from coffee leaves as described previously (Hörtensteiner et al., 1995). RCC was synthesized from methyl-Pheide *a* as reported by Kräutler et al. (1997).

Table 1. *Dependence of Bn-FCC-2 formation from Pheide a on light and reductant*

Washed gerontoplast membranes from canola before solubilization and a stroma fraction (S1) of canola were incubated in the light or in the dark for 30 min at room temperature in the coupled assay described in "Materials and Methods." 100% activity corresponds to 9274 FU of *Bn*-FCC-2 min^{-1} g^{-1} fresh weight.

Incubation Mixture	Conditions	*Bn*-FCC-2
		% of control
Complete	Light	87
Complete	Dark (control)	100
-NADPH, -Glc6P, -Glc6P-DH	Light	69
-NADPH, -Glc6P, -Glc6P-DH, Triton X-100	Light	15
-NADPH, -Glc6P, -Glc6P-DH	Dark	14
-NADPH	Dark	16
-Fd	Dark	1

RESULTS

Requirement for Fd in the Light-Driven Cleavage of Pheide *a* to a Primary FCC

As shown previously, the in vitro production of a primary FCC that is identical in barley (*Hv*-FCC-4) and canola (*Bn*-FCC-2) depends on the presence of reduced Fd in the assay mixture (Schellenberg et al., 1993; Hörtensteiner et al., 1995). Fd is kept in the reduced state by the addition of NADPH, Glc6P, and Glc6P-DH to the assay. FNR, which reduces Fd with NADPH, is present in both enzyme preparations necessary for FCC production, namely the membrane-localized PaO and a soluble stroma protein.

FCC production in washed membranes from chloroplast preparations of senescent canola cotyledons (i.e. before solubilization) could be promoted in the absence of the Fd-reducing system mentioned above when incubations were performed in the light (Table I). The activity was slightly less than that of complete assay mixtures either in the light or in the dark. The light-driven activity still depended on the presence of stroma protein obtained from the first washing step of the chloroplast membranes (data not shown). In addition, integrity of the gerontoplast membranes was necessary for activity, since after solubilization with 1% Triton X-100 for 30 min, the light-dependent FCC production was reduced to only 15%. The same residual activity was observed when either NADPH or the complete Fd-reducing system was omitted. Removal of Fd from a dark-incubated assay completely inhibited *Bn*-FCC-2 production.

Fd-Dependent Formation of Primary FCCs from a Red Bilin

Structural analysis of the first identifiable product of Pheide *a* cleavage in canola, *Bn*-FCC-2 (Fig. 1; Mühlecker et al., 1997), suggests that two atoms of oxygen and four atoms of hydrogen are introduced into Pheide *a* by the combined action of PaO and the stroma protein. The red bilin depicted in Figure 1 was tentatively assumed to represent an intermediary catabolite of the Pheide *a* to FCC conversion (Mühlecker et al., 1997). According to the nomenclature of Chl catabolites proposed by Ginsburg and Matile (1993), it was named RCC.

Incubation of chemically synthesized RCC (Kräutler et al., 1997), together with a preparation of stroma proteins, resulted in the formation of FCCs, provided that reduced Fd was furnished under anaerobic conditions (Fig. 2; Table II). Three fluorescent products could be identified as FCCs by their typical absorption maxima at 320 and 360 nm (Ginsburg and Matile, 1993; Mühlecker et al., 1997; data not shown). According to its retention time on HPLC, peak 2 was found to be identical to *Bn*-FCC-2, whereas the minor peak 3 corresponded to *Ca*-FCC-2, the first identifiable product of Pheide *a* cleavage in the presence of enzyme preparations from ripening fruits of *C. annuum* (Moser and Matile, 1997).

The chemical constitutions of *Bn*-FCC-2 and *Ca*-FCC-2, respectively, turned out to be identical; they are likely to represent stereoisomers at position C1 (see Fig. 1; W. Mühlecker and B. Kräutler, personal communication). The third FCC (peak 1) could not be associated with an authentic FCC; it may also be a stereoisomer, presumably at the C13^2 position of the isocyclic ring. The proportions of the

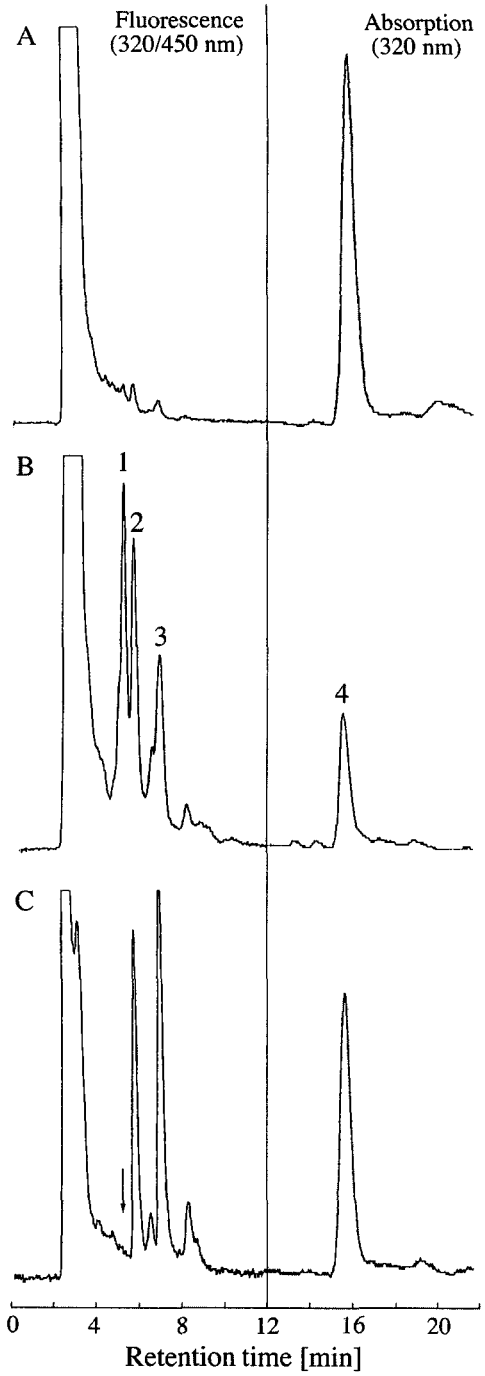

Figure 2. HPLC identification of primary FCCs (1, 2, and 3) as products of RCC (4) reduction catalyzed by stroma proteins isolated from senescent barley leaves before incubation (A) and after 60 min of incubation under anoxic conditions (B). C, Standard solution containing *Bn*-FCC-2 (2), *Ca*-FCC-2 (3), and RCC (4). The arrow in C indicates the position of the unknown FCC (1). Note that the mode of detection switches from fluorescence to absorption after 12 min to identify RCC.

Table II. *Conversion of RCC to FCCs as catalyzed by stroma protein from barley primary leaves*

The complete assay mixtures contained RCC, NADPH, Glc6P, Glc6P-DH, and Fd as detailed in "Materials and Methods." Incubation was for 1 h at room temperature. Products were identified by their retention times on HPLC.

Assay Mixture	Incubation Condition	Product Formation		
		Peak 1	*Bn*-FCC-2	*Ca*-FCC-2
		FU 10^{-2} min^{-1} g^{-1} fresh wt		
Complete	Anoxic	33	31	22
Complete	Air	2	0	0
-Fd	Anoxic	15	17	9
-NADPH	Anoxic	0	0	0
-NADPH, -Glc6P, -Glc6P-DH	Anoxic	0	0	0
Complete +FNR (20 milliunits)	Anoxic	62	45	30

three different FCCs were similar in several independent experiments.

Concomitant with the formation of the three FCCs in the complete assay, the amount of RCC decreased (Fig. 2). When RCC was incubated together with a stroma preparation in the absence of either NADPH or the Fd-reducing system, FCC production could not be detected (Table II). However, FCC formation occurred when only exogenous Fd was omitted. This is due to the fact that the 50 to 80% protein preparation contained FNR as well as Fd (data not shown). The amounts of endogenous Fd and FNR were limiting and, thus, activity was considerably enhanced by supplementing the assay with either Fd or both Fd and FNR.

Fd-Dependent Formation of RCC from Pheide *a*

To investigate whether RCC is truly an intermediate in the conversion of Pheide *a* to FCC, gerontoplast membranes were washed free from adhering stroma proteins, solubilized, and incubated for 60 min in the presence of Pheide *a*, Fd, and the Fd-reducing system (NADPH, Glc6P, and Glc6P-DH). Analysis of the products by reverse-phase HPLC revealed a compound eluting after 15.8 min, the retention time of RCC. It also had the typical absorption maxima at 320 and 485 nm of RCC (Kräutler et al., 1997; data not shown). The compound was absent before incubation and, therefore, we conclude that RCC had been produced.

Unfortunately, under the conditions described, *Bn*-FCC-2 was also produced, presumably because the PaO preparation contained residual stroma protein (Table III). Therefore, proteins of solubilized gerontoplast membranes were subjected to ion-exchange chromatography on EAH-activated Sepharose, resulting in the partial purification of PaO with an increase in specific activity by a factor of more than 100 (data not shown). FCCs were not produced by such a partially purified oxygenase preparation (Table III). However, the formation of RCC during 60 min of incubation was unambiguously detectable when assays were supplemented with FNR (Fig. 3). RCC formation was dependent on the presence of Fd and the Fd-reducing system, since no product was found when Fd, NADPH, or the reducing system was omitted from the assay mixture (Table III). The activity of PaO for the synthesis of RCC was also dependent on the presence of oxygen.

Retention of Fd Protein during Senescence

As shown above and in earlier reports (Schellenberg et al., 1993; Ginsburg et al., 1994), Fd is an indispensable component of Chl breakdown in vitro. In addition, the Fd I gene of maize has been shown to be expressed during leaf senescence (Smart et al., 1995), suggesting that Fd has a senescence-associated function in vivo. We therefore investigated the abundance of Fd protein during senescence of barley primary leaves incubated in permanent darkness.

Table III. *Formation of RCC as catalyzed by partially purified PaO*

Complete assay mixtures contained Pheide a, NADPH, Glc6P, Glc6P-DH, Fd, and FNR as outlined in "Materials and Methods." Incubation was for 1 h at room temperature. RCC was identified by its relative retention time on HPLC.

Incubation Condition	Product Formation	
	Bn-FCC-2	RCC
	FU 10^{-2} min^{-1} g^{-1} fresh wt	*A_{320} 10^{6} min^{-1} g^{-1} fresh wt*
Complete assay[a]	57	59
Complete assay[b]	0	65
-Fd[b]	0	0
-NADPH[b]	0	0
-NADPH, -Glc6P, -Glc6P-DH[b]	0	0
Complete assay, anaerobic[b]	0	0

[a] Solubilized gerontoplast membranes. [b] Partially purified PaO.

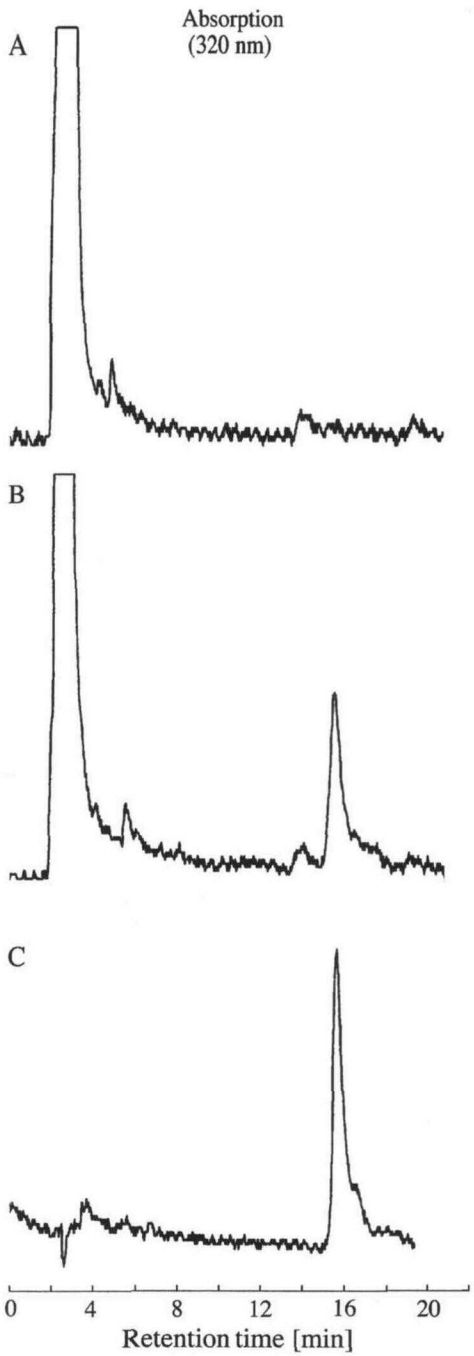

Figure 3. Formation of RCC in an assay containing partially purified PaO from canola and Pheide *a* as a substrate. HPLC identification of reaction mixtures before incubation (A) and after incubation for 60 min in darkness (B). C, Standard solution of RCC; note the absence of the front peak, which in A and B is due to NADPH present in the assay mixtures. RCC eluted after 15.8 min.

Compared with other proteins (e.g. the small and large subunits of Rubisco), the abundancies of which rapidly decreased during the first days of the incubation period (Fig. 4B), Fd was retained until very late stages of senescence (4th-5th d) as judged by signal intensities of western blots visualized with anti-Fd antibodies (Fig. 4A). This result is consistent with the proposal that Fd has a role in processes such as Chl breakdown during senescence.

DISCUSSION

The third step of the catabolic pathway linking up with the hydrolysis of phytol and the dechelation of the central magnesium ion is undoubtedly the most interesting part of Chl breakdown. Not only is it responsible for the loss of green color but it is also unique in comparison with other conversions of porphyrins into linear tetrapyrrolic compounds. The structure of Chl catabolites such as *Hv*-NCC-1 from barley (Kräutler et al., 1991) and *Bn*-FCC-2 from canola (Mühlecker et al., 1997) indicate that cleavage takes place regiospecifically in C4/C5 mesoposition, whereby the C5 carbon of the methine bridge is preserved as a formyl group attached to pyrrol B.

Whereas the regioselectivity of the cleavage is the same in conversions of porphyrins into biliverdin, phytochromobilin, and phycobilins (Beale, 1993; Weller et al., 1996), the methine bridge carbon is lost in these cases. The same selectivity was also found in the red bilin from *Chlorella protothecoides* (Engel et al., 1991, 1996), but another mechanism seems to be responsible for breakdown of Chl into the luciferin of the dinoflagellate *Pyrocystis lunula*, which is oxygenolytically cleaved at the C20/C1 mesoposition (Nakamura et al., 1989).

Apart from such differences of mechanisms of macrocycle opening, there are also similarities such as the redox cycles involved in porphyrin oxygenation. Thus, cyanobacterial phycobilin formation (Rhie and Beale, 1992), as well as breakdown of Pheide *a* in higher plants (Hörtensteiner et al., 1995), depend on Fd as a reductant. In the assay system

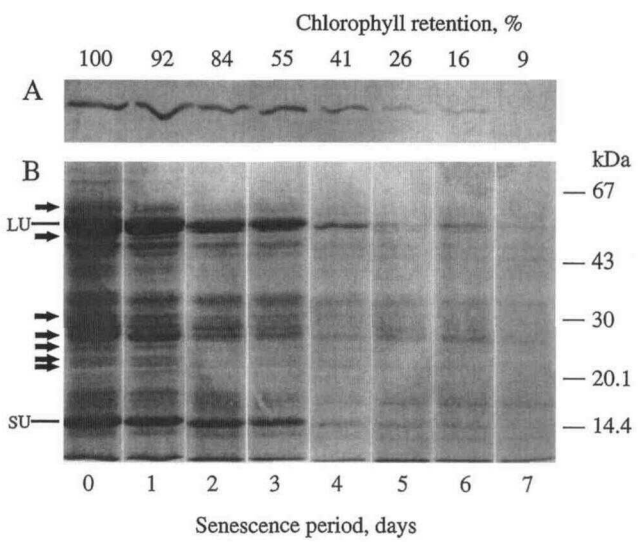

Figure 4. Fd and total protein in senescing barley leaf tissue separated by SDS-PAGE and visualized by immunoblotting (A, Fd) and Coomassie staining (B, total protein). Retention of Chl was measured with a Chl meter (SPAD-502, Minolta). Molecular masses of standard proteins are indicated. Arrows point to proteins that largely disappeared within the first 2 d of senescence. SU, Small subunit of Rubisco; LU, large subunit of Rubisco.

for FCC production from Pheide *a*, NADPH and the NADP-reducing components Glc6P and Glc6P-DH are employed to keep Fd in the reduced state. When employing partially purified PaO, FNR is also required for the transfer of electrons from NADPH to Fd. When unsolubilized membranes were employed for FCC production it was possible to replace NADPH by light. This finding is important with regard to Chl breakdown under natural conditions, i.e. the light-driven reduction of Fd at PSI. Because under these conditions the reaction still depends on the presence of Fd, the finding also demonstrates that Fd, not NADPH, acts as electron donor to the oxygenase. Admittedly, the significance of the light-driven supply of reductants for Chl breakdown under natural conditions cannot be estimated on the basis of results obtained with preparations of gerontoplast membranes.

Using chemically synthesized RCC (Kräutler et al., 1997), it is now possible to demonstrate that the conversion of Pheide *a* into a primary FCC occurs in two steps, which are catalyzed by the membrane-bound oxygenase and the soluble stroma enzyme, respectively (Fig. 5). In the absence of stroma protein, the red bilin RCC emerged as the product of the Fd-driven PaO. RCC did not accumulate, however, presumably because its release from oxygenase requires the presence of the stroma protein. This enzyme transforms RCC to three FCCs, including the FCC previously identified as the primary FCC in canola and in barley.

The reduction of RCC, surprisingly, turned out to also require Fd as an electron donor; therefore, the enzyme may tentatively be named RCC reductase. The three FCCs produced can be identified by their different retention times on reverse-phase HPLC. One of them appears to be identical to the expected and structurally elucidated *Bn*-FCC-2 (Fig. 1; Mühlecker et al., 1997). The second FCC is chromatographically identical to *Ca*-FCC-2, the primary FCC produced from Pheide *a* by enzyme preparations from the pericarp of ripening fruits of sweet pepper (Moser and Matile, 1997). The second FCC differs from *Bn*-FCC-2 only in the absolute configuration at position C1 (W. Mühlecker and B. Kräutler, personal communication).

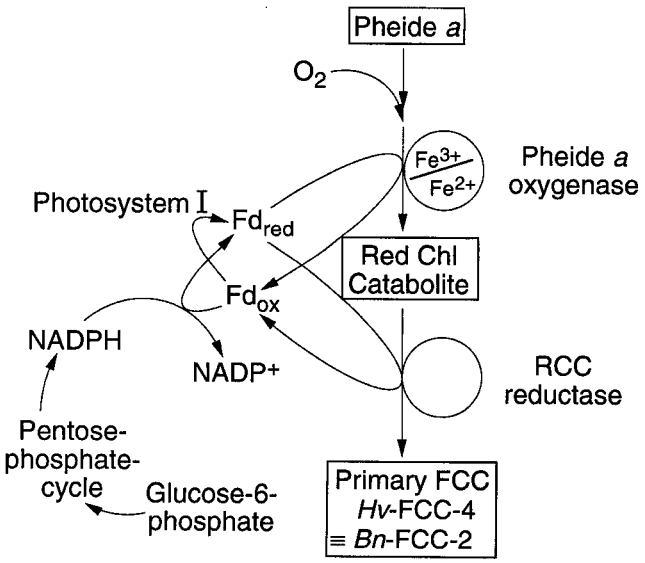

Figure 5. The conversion of Pheide *a* to a primary FCC as catalyzed by Pheide *a* oxygenase in conjunction with RCC reductase.

In the uncoupled reaction, RCC reductase may be able to interact with its substrate, RCC, without spatial restriction by the oxygenase and, therefore, produce FCCs with different configurations, whereas only one product is formed in the coupled assay. In a second report (Rodoni et al., 1997), we demonstrate that in a coupled assay RCC reductases prepared from different plant sources either produce the *Bn*-FCC-2 or the *Ca*-FCC-2 type of primary FCCs. We have not been able to identify the nature of the third and most polar FCC emerging in the uncoupled reaction of RCC reductase. The UV absorption spectrum of this FCC did not allow us to distinguish it from the two FCCs identified.

In vitro the reduction of RCC can be demonstrated only under anoxic conditions. The relevance of this finding for Chl breakdown in vivo is not clear. It is feasible that in the coupled reaction, the action of the oxygen-consuming PaO creates an anoxic microenvironment that favors the subsequent reduction of the intermediary RCC, possibly by preventing the reoxidation of reductant at the catalytic site of RCC reductase.

In contrast to PaO, the activity of which has so far been detected only in senescent leaves of various plant species and in ripening fruits of *C. annuum*, RCC reductase seems to be a constitutive protein that is present in green leaves as well. Its partial purification and characterization will be described in a forthcoming report.

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